The ETS-1 transcription factor has both a hematopoietic intrinsic and extrinsic role in the marginal zone vs. follicular fate decision

A Senior Honors Thesis

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By

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Abstract

Marginal Zone (MZ) B cells develop in the spleen from immature transitional B cells. The molecular mechanisms underlying the development of MZ B is largely unknown. Ets-1 is a member of the Ets proto-oncogene family of transcription factors that plays diverse roles in the immune system. MZ B cells do not develop in the absence of Ets-1.

The role of Ets-1 in the development of the MZ B cells is studied by comparison of splenic subpopulations leading to the MZ fate by FACS analysis of Ets-$1^{-/-}$ and Wild type mice. Ets-$1^{-/-}$ mice exhibited statistically significant decreases in Marginal Zone Progenitors and Marginal Zone Progenitor Transitional cells and the development of an $IgD^{lo}CD21^{mid}CD23^{hi}$ population, traditionally thought to be Follicular B cells when compared with wild type mice. The nature of Ets-1's role in the development of MZ B cells was determined through an adoptive transfer experiment. The adoptive transfer suggested hematopoietic intrinsic and extrinsic roles for Ets-1 in the development of MZ B cells. The defective subpopulations leading to MZ B cells all showed statistically significant results suggesting a hematopoietic intrinsic role of Ets-1. Analyzing the MZ B cell population showed the same intrinsic role accompanied with significant results also suggesting a minor extrinsic role. Studies analyzing the reversal of an activated phenotype on B cells have shown Ets-1 to work in a hematopoietic extrinsic manner. Thus, Ets-1 may play a multi-level regulatory role in the development and activation of B cells. Better understanding the role of Ets-1 in MZ B cell development will allow us to better understand the origin of the disease and design targeted therapeutics.

Very little is understood about Ets-1 signaling as well as MZ B cell development. An Ets-$1^{-/}$ hCD37 transgenic mouse allowed study of the interaction between the cell surface CD37 receptor and Ets-1. CD37 is a tetraspanin protein highly overexpressed in malignant B cells. Tetraspanins are associated with an array of biological processes including cell activation, survival, proliferation, adhesion, and migration, making it a great tool to use to further study Ets-1's role in MZ B cell development (1). Overexpression of CD37 altered the splenic B cell subpopulation distribution in Ets-1 mice, indicating that the two proteins have an inter-related role.

Acknowledgements

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Introduction

Our body's immune system protects us from invading pathogens and other harmful threats from our environment. The second largest immune organ is the spleen (2). The spleen is responsible for filtering blood for foreign material and damaged red blood cells along with initiating immune responses to blood-borne antigens (2).

The development of the embryonic spleen proceeds through the preliminary, transformation, and a primary follicle stage (3). The preliminary stage begins at 14 gestational weeks, gw and is marked by the beginning of hematopoiesis and erythropoiesis (3). A week later, at 15 gw, the splenic lobules and red pulp begin forming around lobules at the transformation stage (3). At 23 gw, primary follicles lead to the formation of the B cell region (3).

In the spleen, most B cells home to the follicles of the splenic white pulp (4). These cells are follicular B cells. Follicular B cells work closely with helper T cells and are involved in T cell dependent immune responses to protein antigens (4). The innate like B cell population that transitions between the marginal zone and the follicles are called Marginal Zone (MZ) B cells (5). MZ B cells are an important source of lipid specific antibodies and have the ability to self-renew and survive as long as the host (4). An expansion of MZ B cells have shown to cause autoimmune pathogenesis (5). For example, NOD mice display an increase in MZ B cells and end up with Type 1 Diabetes and Sjögren's syndrome (5). Gene targeted mic studying the transcription factor Klf2 show that altered MZ migration lead to the gain of function of follicular B cells to respond to MZ associated antigens and pathogens in a T *de*pendent manner (6).

Cells enter the spleen and undergo multiple stages of development before becoming a follicular or MZ B cell. They go through the T1, T2, T3 cell stages before becoming a follicular B cell (4), or the T1, Marginal Zone Progenitor Transitional 1 (MZP T1), Marginal Zone Progenitor (MZP) cell stage before becoming a MZ B cell (7). There is also a developmental pathway into the MZ B cell fate through an IgM^{hi} follicular stage called follicular II cells (4). In the absence of Rac1 and Rac2, transitional B cells arrest at an IgD^{-} stage known as T0 (8). The cell must be a T1 cell before it can home to the white pulp of the spleen (8). Rac1/2 GTPase, important for cell migration, adhesion, proliferation and survival, is required for B cells to develop from the T0 to T1 stage (8). This same phenotype is seen with $Syk^{-/-}$, showing a developmental checkpoint that coincides with B cell positive selection (8). Developing into a MZ B cell requires NF-ϰB activation through activation of BAFF, from the TNF family receptor (5). A weak BCR signal leads the B cell into the Marginal Zone while a strong BCR signal leads the cell to the follicular B cell state (4). Increasing the strength of the BCR signal, however, will result in apoptosis of the follicular B cell (4). Notch2 and other migration mediated signaling are also thought to be involved (5). DL1 is a notch signaling ligand that is found in splenic venules, but not in the bone marrow, involved in MZ B cell development (4).

Understanding the development of MZ B cells is critical because of the importance this small subset of cells play in the immune system, shown by their longevity throughout the host's lifetime. Information regarding the development of MZ B cells could lead to treatment of autoimmune diseases such as type 1 diabetes, Lupus erythematosus, and even cancers. Most genes that are known to be involved in deciding the MZ vs. follicular B cell fate are signaling ligands or proteins involved in migration. Regulatory proteins such as transcription factors have not yet been highly studied, other than Klf2 (6) which is also involved in migration. Ets1 is a

developmentally critical gene that may play a more defining role in the MZ vs follicular B cell fate decision.

The Ets-1 gene belongs to the Ets proto-oncogene family and plays diverse roles in the immune system as a transcriptional factor[1,2,3]. The Ets family has been conserved throughout the Metazoa (9), but are not found in fungi, plants or protozoans (9). Ets-1 is also a developmentally critical gene that is predominantly expressed lymphoid organs in both neonatal and adult mice (10), especially in B and T cells of adult mice (I). Ets-1 is first highly expressed in embryos after implantation and during organogenesis (10). At this point, Ets-1 is expressed in all organs of the 15 day old embryo (10). In later fetal stages, Ets-1 expression reduces in organs such as the stomach or intestines (10) and becomes confined mainly to brain, lymphoid tissues, and organs undergoing branching morphogenesis (10), such as the lung.

Knockout of Ets-1 leads to defects in T cell activation (11), defects in NK cell development (12), and defects in differentiation or regulation of B cell (11, 13). High level of Ets-1 expression tends to be confined to lymphoid organs, which implies involvement in development or functional differentiation of lymphoid cells (14). Because Ets-1 has been known to be highly involved in the development of cells of the lymphoid lineage, this project explored its role in the Marginal Zone vs Follicular fate decision of B cell splenocytes.

Materials and Methods

Generation of Ets-1$^{-/-}$ mouse

The Ets-1$^{-/-}$ mice were generated using a target mutation of embryonic stem cells at the OSU CCC/CRI Transgenic Animal Shared Resource center. Targeted 129 ES cells were injected into C57Bl/6 blastocysts and then Sv/C57Bl/6 chimeric mice were bred to C57Bl/6 mice to make heterozygous Ets-1 mice (Ets-1$^{+/-}$). These were then interbred to make Ets-1$^{-/-}$ and Ets-1$^{+/+}$ mice. Genotype had been determined by southern blot and the knockout phenotype was confirmed by western blot with an anti-Ets-1 antibody. All experiments were done with Ets-1 knockout mice and Ets-1 wildtype mice on 129 Sv/C57Bl/6 background. Mice were all sacrificed using primary, CO_2 asphyxiation, and secondary, cervical dislocation, methods. All mouse handling and experimental procedures were carefully performed under guidelines of an approved Institutional Animal Care and Use Committee protocol (IACUC). The genotype of the mice used were confirmed using DNA primers and Polymerase Chain Reaction (PCR) techniques.

Processing of splenocytes

Spleens removed from mice were stored in PBS and then processed within 6 hours or less. Spleens were mashed by using the end of a 10 mL syringe plunger to gently grind the spleen against a 50 μm filter placed in 6 well plates, kept sterile and performed in a biosafety cabinet hood. These 6 well plates were filled with RPMI medium 1640 (Gibco). Once the cells contained in the spleen were released into the solution,

Flow cytometric analysis of splenic B cell subpopulations

Processed splenocytes were distributed into flow tubes and stained at a concentration of 4e7 cells/mL with anti-Mouse IgM (bv510 Rat anti-Mouse IgM, BD Horizon), IgD (anti-Mouse

IgD PE-Cy7, eBioscience), B220 (anti human/mouse CD45R (B220) APC-eFluor780, eBioscience), CD23 (anti-Mouse CD23 FITC, eBioscience), CD21 (APC Rat anti-Mouse CD21/CD35, BD Pharmingen), CD1d (Percp/Cy5.5 anti-Mouse CD1d (CD1.1, Ly-38), BioLegend), AA4.1 (PE Rat anti-Mouse Early B Lineage Clone AA4.1, BD Pharmingen) and Propidium Iodide. Volume of each antibody used was optimized and titrated, so different amounts of each antibody was used, however, the volumes and final staining concentrations were kept constant across samples and across experiments. All flow cytometry experiments were performed on the Gallios Flow Cytometer (Beckman Coulter). Analysis of flow was done using Kaluza software. Gates were set using FMOs where appropriate (details about gating strategy found in figure legend and supplemental data).

Adoptive transfer experiment

The adoptive transfer experiment had 3 experimental groups (Figure 5). At 6-8 weeks of age, $1x10^7$ Ets-$1^{-/-}$ bone marrow cells were engrafted into sub-lethally irradiated congenic mice (n=9). For the last two groups, $1x10^7$ congenic bone marrow cells were engrafted into sub-lethally irradiated Ets-$1^{-/-}$ mice (n=9) and into sub-lethally irradiated wild-type mice (n=9). After allowing the engrafted cells to reconstitute for approximately three months, their spleens were analyzed by flow.

Results

Marginal Zone B cell loss occurs concomitantly with development of unique B cell populations in the Ets-1$^{-/-}$ spleen

Previous studies have looked at the marginal zone in Ets-1 deficient mice using only the CD21 and CD23 markers in flow cytometry (14). This study incorporates the traditional CD21 and CD23 markers along with other markers such as the early B cell lineage marker AA4.1, the non-classical MHC protein, CD1d along with IgM, IgD and B220. This panel of extensive markers allowed a more holistic review of the distributions of various B cell subpopulations residing in the spleen. The flow gating strategies used included a straightforward gating strategy along with means of verifying the populations with additional markers (Figures S1 and S2). A statistically significant decrease is seen across MZ, MZP and MZP-T1 populations in the spleen (Figures 1A, 2). A moderate decrease is also seen in the T2/T3 B cell populations while there is little difference seen in T1 and follicular B Cell populations (Figure 1A and 2). Along the developmental pathway of MZ B cells (4), a decrease is evident across all populations after the T1 stage without any significant changes in the two follicular stages (Figure 1B). There is also an increase in a population falling within the follicular gate with an abnormal IgD^{lo} status (B220^{+} CD23^{+} CD21^{mid} IgD^{lo}) (Figure 4A) and the unidentified populations with a CD23^{-} CD21$^{lo/-}$ identity (Figures 2 and 4B).

The selective loss of Marginal Zone B cells from splenic lymphocytes is largely a cell intrinsic defect

Results from the adoptive transfer experiment investigating the nature of Ets-1's role in MZ B cell development (Figure 5) showed that there were both strong hematopoietic intrinsic

role and a weak hematopoietic extrinsic role of Ets-1 in the development of MZ B cells. The defective subpopulations leading to MZ B cells all showed statistically significant results suggesting a hematopoietic intrinsic role of Ets-1 (Figures S3 and 6). Analyzing the MZ B cell population showed the same intrinsic role accompanied with significant results also suggesting a minor extrinsic role. Results suggest that the decrease in T2/T3 cells may be driven by a hematopoietic intrinsic effect through an observed increase in the WT to Ets-$1^{-/-}$ mouse compared to the irradiation control. Since, when there is Ets-1 present in the host, less development of T2/T3 cells is seen and when there is Ets-1 present in the donated bone marrow cell more T2/T3 cell is seen, it is possible that Ets-1 plays a negative regulatory role in the non-hematopoietic microenvironment while it plays a positive regulatory role within the hematopoietic cells in the development of T2/T3 cells. The immature stage before Marginal zone precursor is MZP-T1 (a stage in between the transitional 1 and MZP stages). Evaluation of this population as well, shows the same hematopoietic intrinsic differences between the $Ets1^{-/-}$ to WT group and WT to WT but fail to show the extrinsic differences between the WT to WT and WT to $Ets1^{-/-}$ seen in MZ cells. Similar data is shown in the MZP B cell populations. This suggests that heavy hematopoietic intrinsic and moderate hematopoietic extrinsic factors affect the ability of the B cell to develop into a mature MZ cell. One peculiar finding resulting from the adoptive transfer experiment, was that the IgD^{lo} subset of Follicular B cells identified in the Ets-$1^{-/-}$ mouse was found to be absent in both experimental groups as well as in the control groups. Previous studies analyzing the reversal of an activated phenotype on B cells have shown Ets-1 to work in a hematopoietic extrinsic manner (14). Taken together with our data, this suggests that Ets-1 may play a regulatory role in the development and activation of B in a multi-level manner.

Overexpression of CD37 alters the Marginal Zone B cell defect phenotype in Ets-1 knockout mice

In the Ets-$1^{-/-}$ mouse, there is a pronounced increase in the number of $CD21^{lo}/CD23^{-}$ population (Figure 4b). A human CD37 transgenic mouse was crossed with an Ets-$1^{-/-}$ mouse, creating an Ets-$1^{-/-}$ mouse with overexpressed CD37. In this mouse, the $CD21^{lo}/CD23^{-}$ population phenotype is amplified even more greatly than in the Ets-$1^{-/-}$ alone (Figure 8b). CD37 overexpression does not, however, rescue the loss of MZ, MZP or MZP-T1 B cells (Figures 8d, e and f). Overexpressing CD37 also introduces a new phenotype in the Ets-$1^{-/-}$ mouse, by increasing the number of T1 B cells (Figure 8a). Ets-$1^{-/-}$ coupled with CD37 overexpression leads to the loss of follicular B cells (Figure 8c). These three pieces of evidence, together suggests a previously unidentified relationship between the Ets-1 transcription factor and CD37 tetraspanin marker.

Discussion

The mice used in our study, deficient in the Ets-1 protein, had a previously reported loss of marginal zone B cells (14), which normally make up about a tenth of the wild-type B cell population. Hypothesizing that the splenic B cell subpopulations numbers leading the marginal zone B cell fate would also experience disturbances through the deletion of Ets-1, a flow panel that allows examination of transitional B cells, follicular B cells and progenitor populations was optimized. This was important to allow identification of the stage(s) at which Ets-1 plays a role in the MZ B cell development defect. There was a consistent decrease in transitional and precursor populations leading to the MZ B cell fate *after* the T1 stge. Two populations absent in the wild-type mouse were also identified: an IgD^{lo} subset of follicular cells ($CD21^{mid}$ $CD23^{+}$) and a $CD21^{lo}$ $CD23^{-}$ population of B cells. The development of the $CD21^{lo}$ $CD23^{-}$ population suggests improper maturation of the T1 population (e.g., the T1 population loses its AA4.1, immature B cell, surface marker but does not acquire proper signal or is otherwise incapable of transitioning to the T2 or MZP-T1 stage, so becomes a degenerate $CD21^{lo}$ $CD23^{-}$ population).

Researchers have previously studied an Ets-$1^{p/p}$ mouse, (14) which was disrupted along the pointed domain of the Ets-1 gene. Their study found an activated phenotype on the follicular B cells of the Ets-$1^{p/p}$ mouse. Upon further study they saw that this activated phenotype was cell extrinsic (14), with the phenotype reversing in an adoptive transfer experiment. The ability of hematopoietic stem cells to both self renew and to differentiate into different cell types is tightly regulated by the cells and molecules in their immediate environment and 'niche' that they inhabit (15). And so, there was good reason to believe that the loss of MZ B cells was also largely dictated by a hematopoietic extrinsic mechanism. An adoptive transfer experiment was used to see if MZ B cells would be rescued suggesting that hematopoietic extrinsic mechanism. Looking

at MZ B cells, the data suggested mostly a hematopoietic intrinsic role concomitant with a slight, but statistically significant, hematopoietic extrinsic role. Interestingly, this hematopoietic extrinsic role is absent in the two previous precursor populations, MZP and MZP-T1 B cells. Together, these two pieces of data suggests that Ets-1 may play an extrinsic role at the MZP to MZ B cell transition and a hematopoietic intrinsic role after the T1 stage (Figure 9). This is supported by the fact that the $CD21^{lo}$ $CD23^{-}$ population also sees results suggesting a cell intrinsic effect, though not statistically significant (data not shown). A cell intrinsic effect beginning at this stage is in line with the proposed idea that cells do not receive needed signals to mature past the T1 stage and so become incompetent cells not expressing high levels of either CD21 or CD23 (Figure 1b).

In an Ets-1 knockout mouse, there is normally a decrease in the Transitional 2/3 B cell population. In the adoptive transfer, however (Figure 6A, S3) the T2/T3 population in the group with Ets-$1^{-/-}$ cells engrafted into wild-type experienced an increase in the number of cells in the population. This suggests a hematopoietic extrinsic role of ets-1 at the T1 to T2/T3 maturation stage. This information maintains a hematopoietic intrinsic role for Ets-1 as well, since the Ets-$1^{-/-}$ mouse (deficient in Ets-1 cell intrinsically as well as in the microenvironment) alone does not see an increase in this population. Conversely, the follicular B cell population, which does not see high variance in an Ets-$1^{-/-}$ mouse compared to Ets-$1^{+/+}$, the group with Ets-$1^{-/-}$ cells engrafted into a wild-type host shows an increase in the follicular B cell population in male mice (data not shown). This suggests for an intrinsic repressive effect that is seen only in males. For almost every population, the data was more consistent among males than among females. The other population that doesn't show a substantial difference between Ets-1 knockout and wild-type mice is the Transitional 1 B cells. Wild-type T1 cells experienced a decrease in population number

when engrafted into an Ets-1$^{-/-}$ microenvironment (data not shown). This suggests that Ets-1 plays a cell extrinsic role at the T0 to T1 stage, or the stage where the transitional B cell homes to the spleen. The IgD^{lo} subset of follicular B cells seen in the Ets-1 knockout mouse is not seen in any experimental group in the adoptive transfer experiment. The development of that unique population can only be seen in mice with an Ets-1 deficiency in both the cell and the microenvironment, indicating that the presence of Ets-1 in either the cell or the microenvironment is sufficient to prevent the aberrant expansion of this population.

Ets-1$^{-/-}$ mice have a unique morphology of the spleen, so splenic sizes and weights were taken but there were no glaring variations between the three experimental group populations (data not shown). The next step in exploring Ets-1's mechanism of action is to study proliferation ability, in situ cell death and earlier B cell development (evaluate populations beginning in the bone marrow).

CD37 is a tetraspanin marker within the hematopoietic lineage. Tetraspanins work with various proteins promoting many important biological functions of the cell including activation, survival, proliferation, migration and adhesion (1). Not too much is known about Ets-1's signaling pathways but a relationship between the Ets-1 transcription factor and the CD37 surface marker was established with the double mutant mouse. Previous studies (16) have shown that Ets-1 and other ETS family members such as PU.1 play regulatory roles with CD53, also a tetraspanin expressed in lymphoid- myeloid lineages. Because of this previously established relationship, the Ets-1$^{-/-}$ hCD37tg mouse was created to study possible relationships between these two gene products. The next step in furthering this study would be to create a CD37$^{-/-}$ mouse to cross with the Ets-1$^{-/-}$ to better understand the role of the CD37/Ets-1 interaction in the development of MZ B cells. Ets-1 acts in a hematopoietic intrinsic manner after the transitional 1

stage and a hematopoietic extrinsic manner after the marginal zone progenitor stage and has an interrelated role with CD37 in the marginal zone vs. follicular fate decision.

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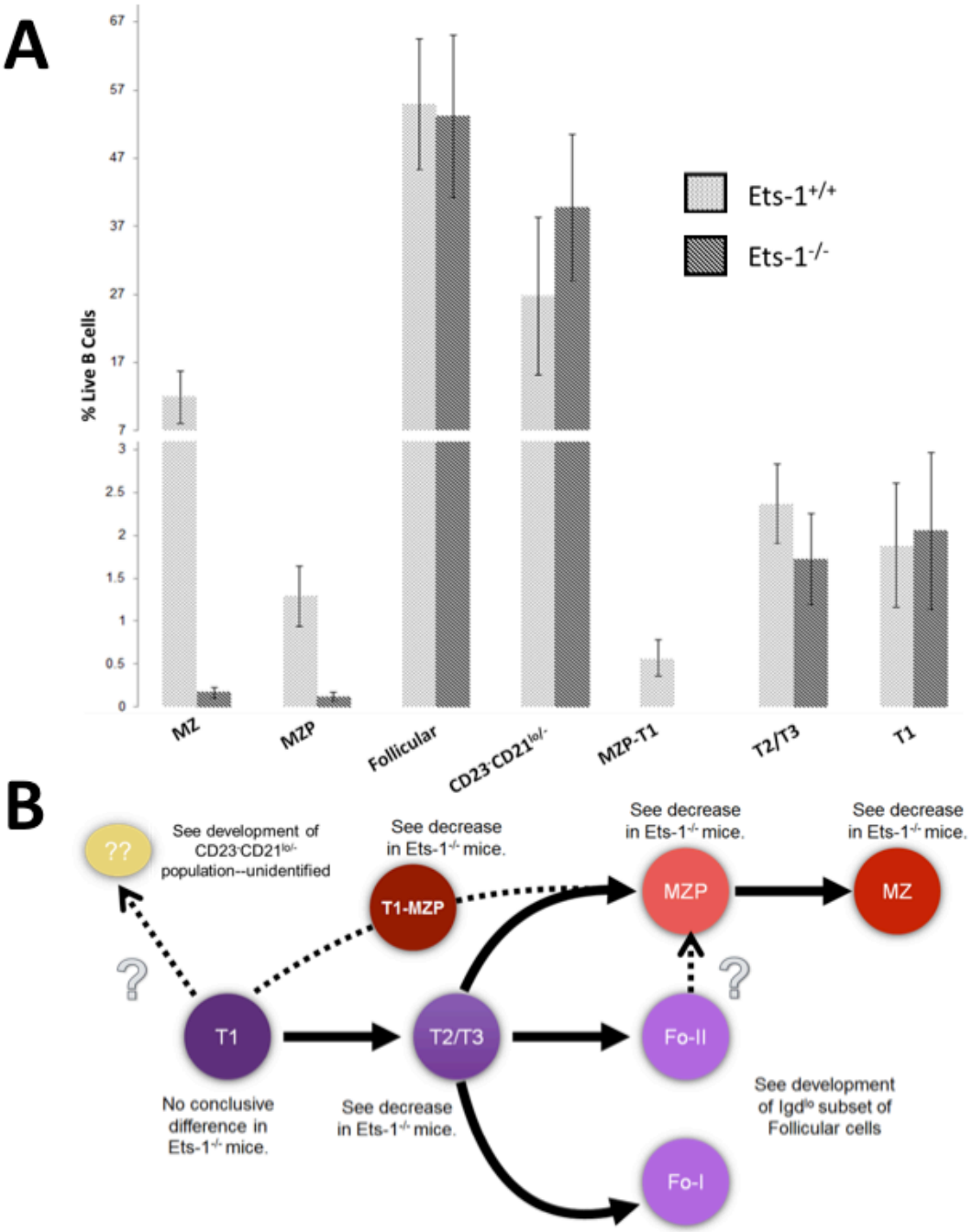
Figures and Supplemental

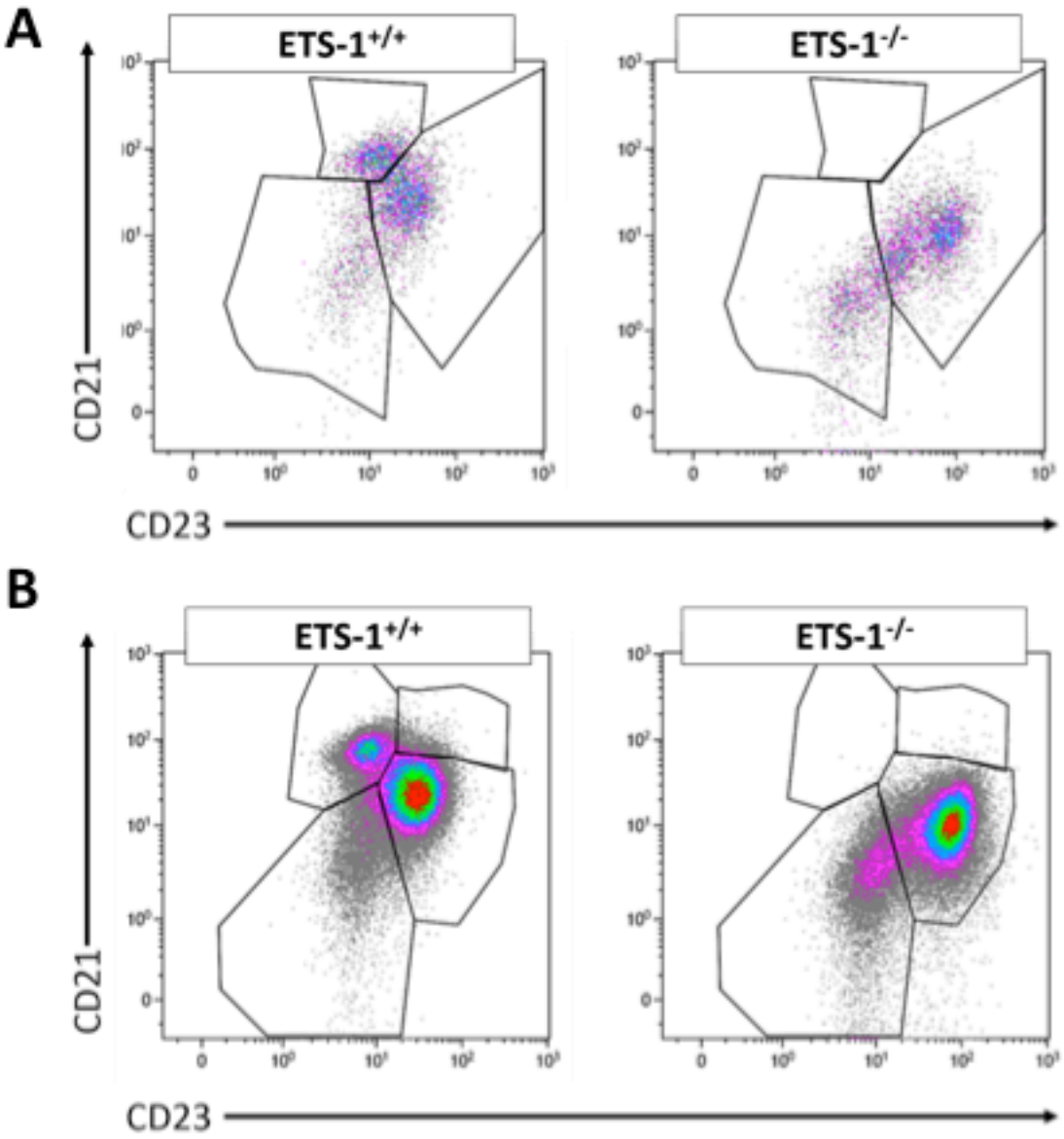
Figure 1: Shows variations across numerous splenic B cell subpopulations.



A.) Bar graph shows increases and decreases in populations: Marginal Zone ($B220^{+}$ $AA4.1^{-}$ $CD23^{-}$ $CD21^{hi}$ IgM^{hi} $CD1d^{hi}IgD^{lo}$), Marginal Zone Progenitors ($B220^{+}$ $AA4.1^{-}$ $CD23^{+}$ $CD21^{hi}$ IgM^{hi} $CD1d^{hi}IgD^{hi}$), Follicular ($B220^{+}$ $AA4.1^{-}$ $CD23^{+}$ $CD21^{mid}CD1d^{hi}IgD^{hi}$), $CD23^{-}$ $CD21^{lo/-}$

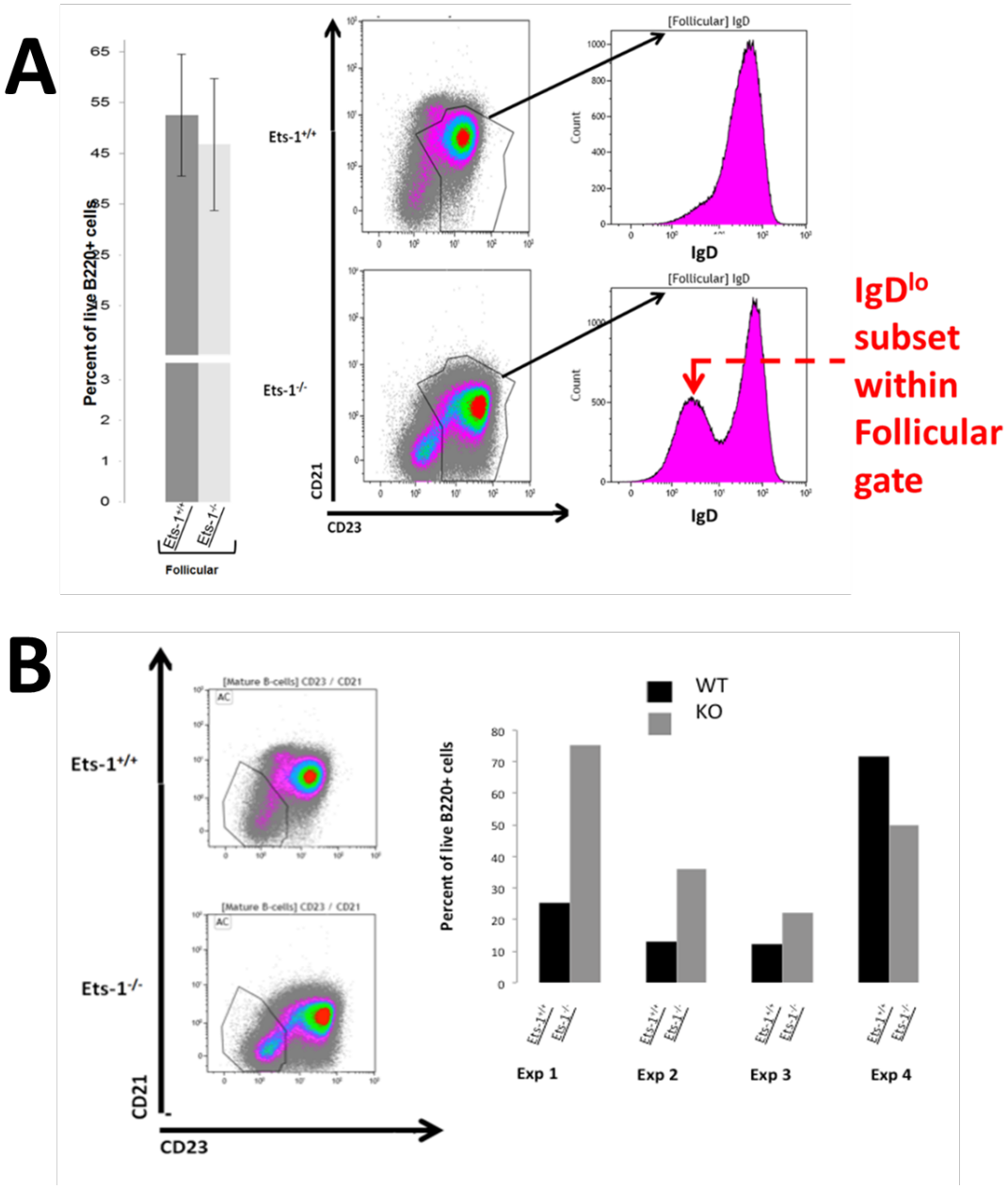
(B220^{+} AA4.1^{-} CD23^{-} CD21$^{lo/-}$), Marginal Zone Progenitors (B220^{+} AA4.1^{-} CD23^{+} CD21^{hi} IgM^{hi} CD1d^{hi}IgD^{hi}), Marginal Zone Progenitors-Transitional 1 (B220^{+} AA4.1^{+} CD23^{+} CD21^{hi} IgM^{hi} CD1d^{hi}IgD^{hi}), Transitional 2/Transitional 3 (B220^{+} AA4.1^{+} CD23^{+} CD21^{mid}), Transitional 1 (B220^{+} AA4.1^{+} CD23^{-} CD21^{lo}) B.) Transitional 1 B cells develop into Marginal Zone cells through multiple pathways, including through a Transitional 1-Marginal Zone Progenitor intermediate, the Transitional 2/3 intermediate or through a Follicular 2 intermediate. We see a decrease in cell subpopulations along every step of this pathway following the Transitional 1 stage. The loss of these populations are accompanied by the development of the CD23^{-}CD21$^{lo/-}$ populations and the development of an IgD^{lo} subset of cells falling into the Follicular gate (B220^{+} CD23^{+} CD21^{mid}). (4, 7)

Figure 2: Flow cytometry data of splenic B cell subpopulations.



Splenocytes were incubated with different antibodies that were used to identify the various splenic B cell subpopulations. Representative flow data shown in the identification of various splenic B cell subpopulations. A.) AA4.1+ populations including MZP-T1 ($CD23^{+}$ $CD21^{hi}$), T1 ($CD23^{-}$ $CD21^{lo}$) and T2/T3 ($CD23^{+}CD21^{mid}$). B.) AA4.1- populations include MZ ($CD23^{-}$ $CD21^{hi}$), MZP ($CD23^{+}$ $CD21^{hi}$), Follicular ($CD23^{+}$ $CD21^{mid}$) and the $CD23^{-}$ $CD21^{lo/-}$ populations.

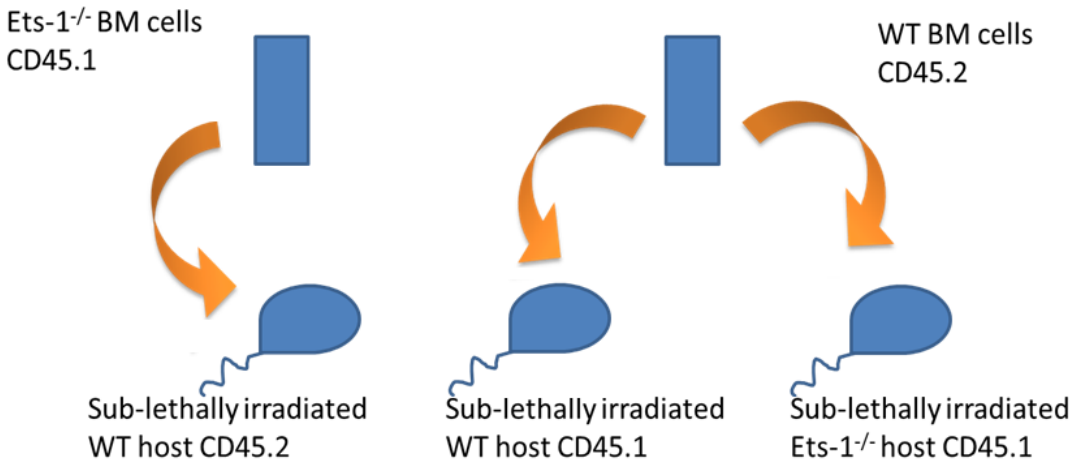
Figure 4: The absence of Ets-1 leads to the aberrant development of two populations.



A.) The number of follicular cells seems to stay static even in the absence of Ets-1. However, a subset of similar cells with an IgD^{lo} status begins to develop in Ets-$1^{-/-}$ mice. These are not properly forming Follicular cells because Follicular cells are defined to have high levels of IgD surface expression. B.) In Ets-$1^{-/-}$ mice, we observe a novel increase in $CD23^{lo/-}CD21^{lo/-}$

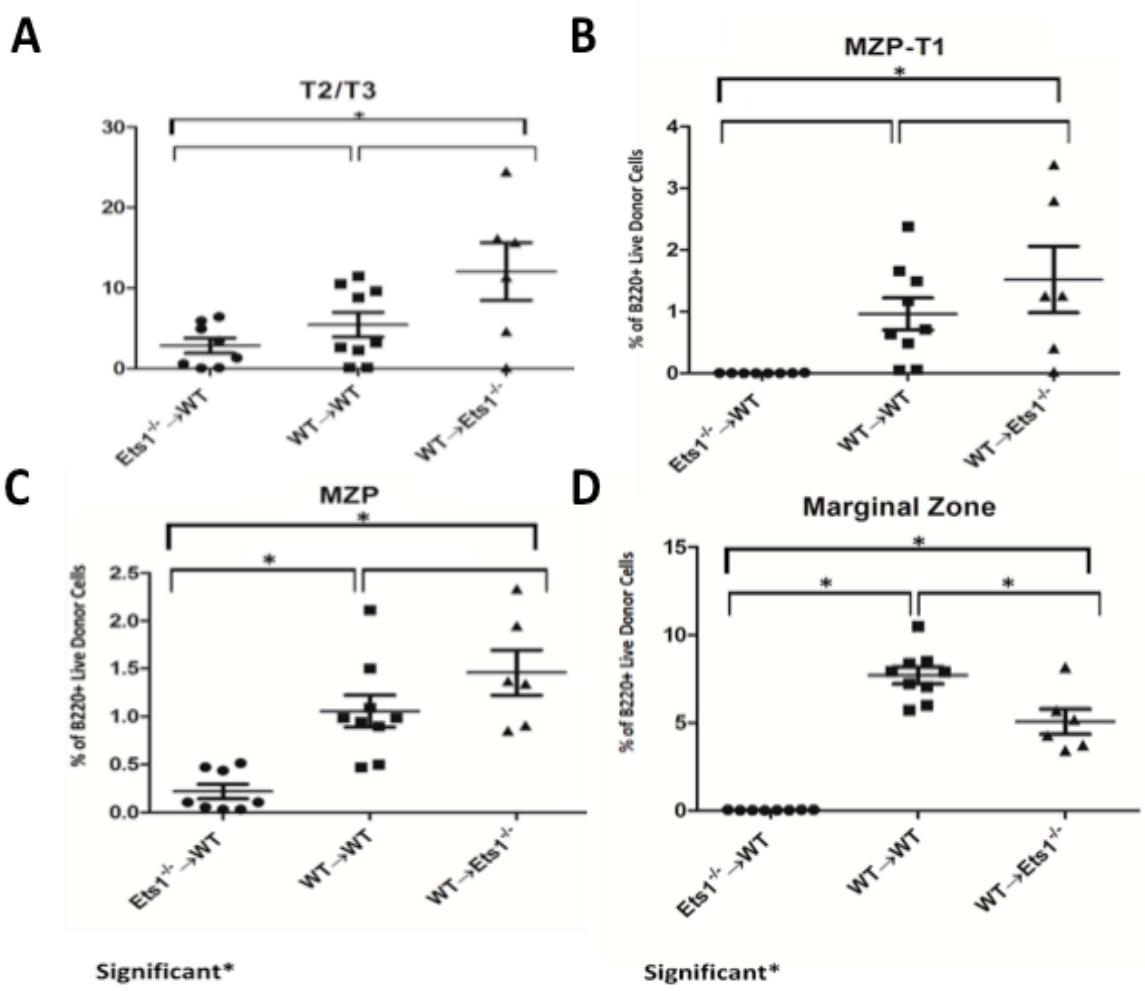
subpopulations in mature B cells ($B220^{+}$ $AA4.1^{-}$). These cells have been unidentified as of yet. Transitional 1 cells are the only cells that share the $CD23^{lo/-}CD21^{lo/-}$ phenotype but they are $AA4.1^{+}$ cells, immature. These could potentially be cells rerouted from the T1 state that do not develop properly into any of the standard mature subpopulations, but lose the AA4.1 marker without gaining any other markers.

Figure 5: Adoptive transfer experiment investigating the nature of Ets-1's role in MZ B cell development.



In order to investigate whether the role of Ets-1 is hematopoietic intrinsic or extrinsic, an adoptive transfer experiment was performed. At 6-8 weeks of age, $3x10^6$ cells from Ets-$1^{-/-}$ CD45.2 and WT CD45.1 mice were engrafted via tail vein injection into sub-lethally irradiated hosts, as shown. Splenic B cell subpopulations were analyzed by flow cytometry after a 3 month reconstitution period.

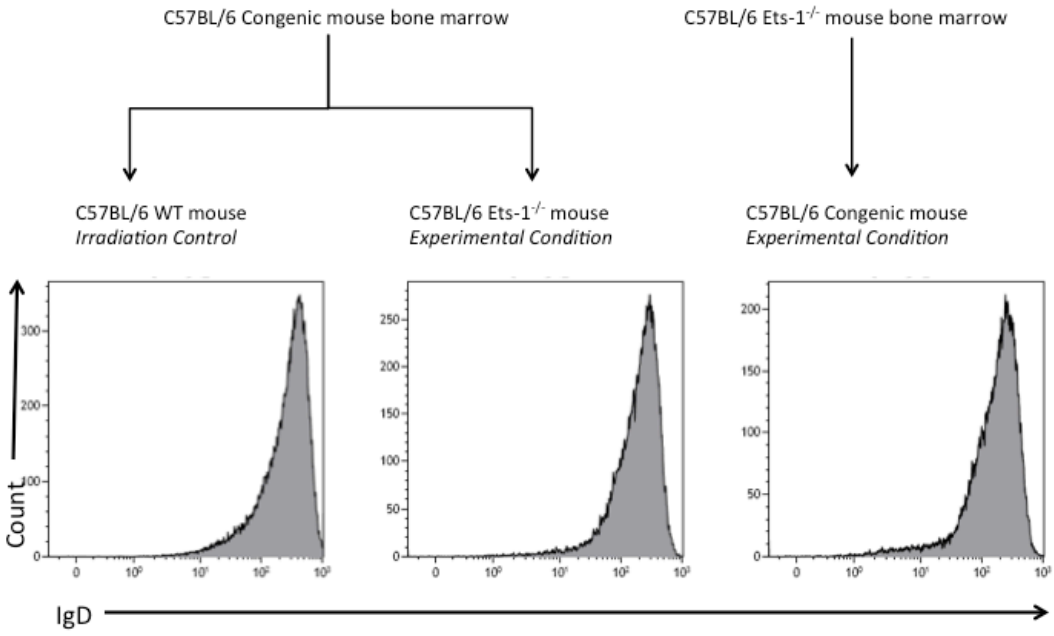
Figure 6: Adoptive transfer results from T2/T3. MZP-T1, MZP and Marginal Zone cell subpopulations.



When analyzing after three months, results suggested heavy intrinsic role of Ets-1 and some degree of extrinsic role as well. A.) Results suggest that the decrease in T2/T3 cells may be driven by a hematopoietic intrinsic effect through an observed increase in the WT→Ets-$1^{-/-}$ mouse compared to the irradiation control. Since, when there is Ets-1 present in the host, less development of T2/T3 cells is seen and when there is Ets-1 present in the donated bone marrow cell *more* T2/T3 cell is seen, it is possible that Ets-1 plays a negative regulatory role in the non-

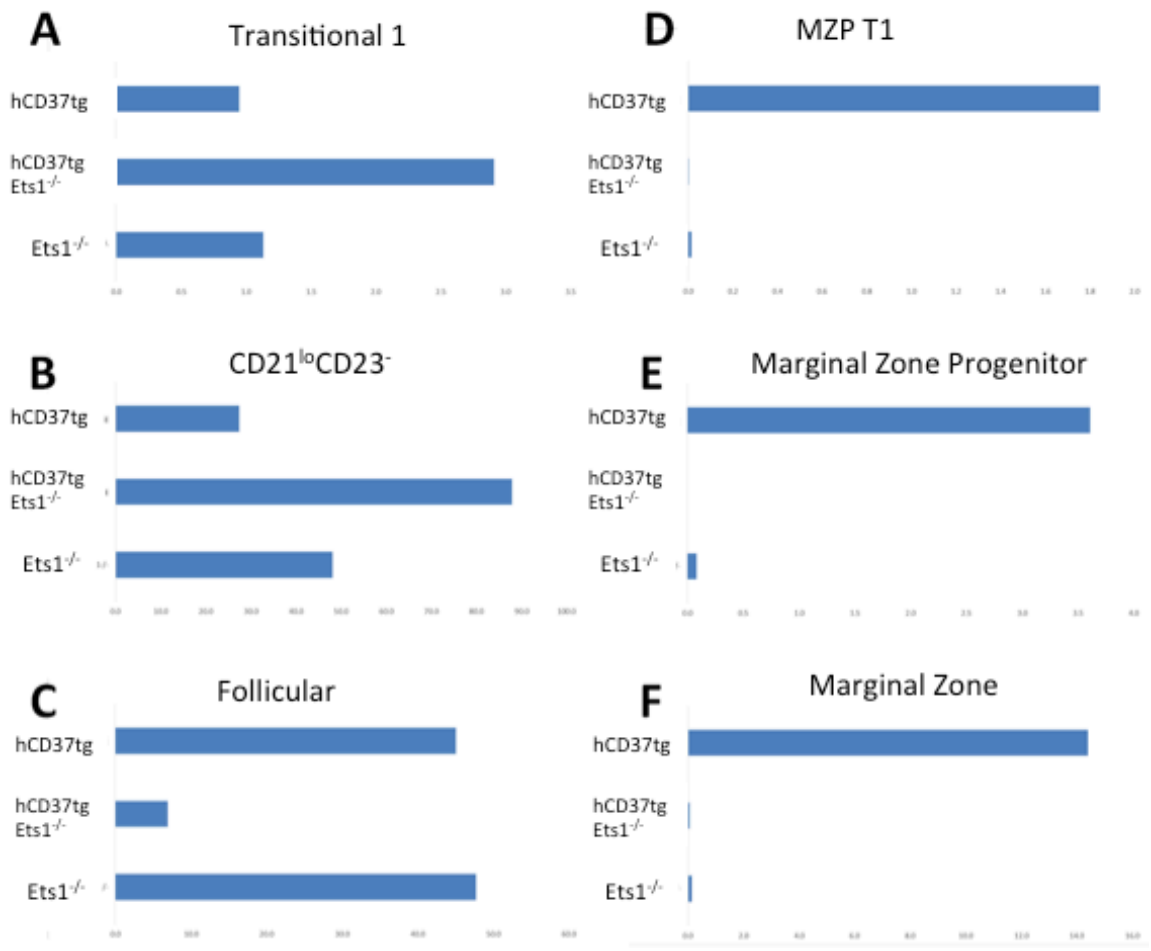
hematopoietic microenvironment while it plays a positive regulatory role within the hematopoietic cells in the development of T2/T3 cells. B.) The immature stage before Marginal zone precursor is MZP-T1 (a stage in between the transitional 1 and MZP stages). Evaluation of this population as well, shows the same hematopoietic intrinsic differences between the $Ets1^{-/-}$ →WT group and WT→WT but fail to show the extrinsic differences between the WT → WT and WT → $Ets1^{-/-}$ seen in MZ cells. C.) Marginal Zone Precursors, which are one step before MZ B cells, show the same hematopoietic intrinsic differences but fail to show the extrinsic differences seen in Marginal Zone cells. D.) In the marginal zone populations, you see a statistically significant difference between all three experimental groups, though visibly, it is quite obvious that the difference between the $Ets1^{-/-}$→WT group and WT→WT is much greater than the difference between WT→WT and WT→$Ets1^{-/-}$. This suggests that heavy hematopoietic intrinsic and moderate hematopoietic extrinsic factors affect the ability of the B cell to develop into a mature MZ cell.

Figure 7: Adoptive transfer results of IgD^{lo} subset of Follicular B Cells



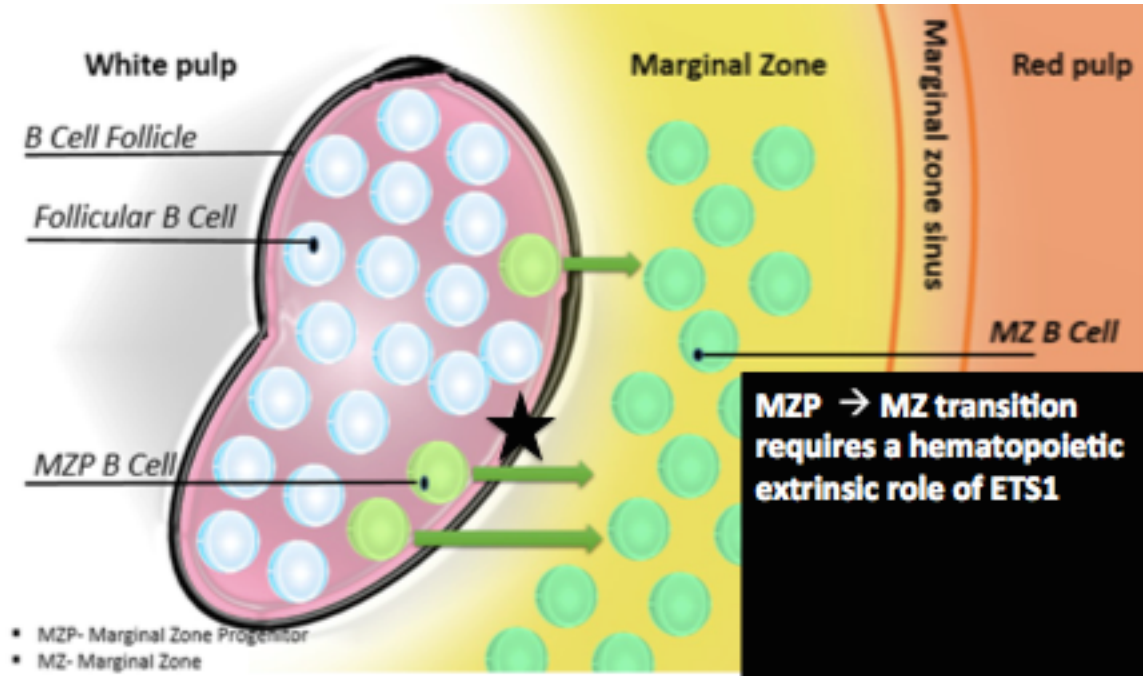
Histograms are gated on follicular cells (CD23^{+}CD21^{mid}AA4.1^{-}B220^{+}). The increased CD23^{+} CD21^{mid}IgD^{lo} population phenotype of the Ets-1$^{-/-}$ did not occur in any of the adoptively transferred mice. This suggests that Ets-1 in *either* the microenvironment or intrinsically within the cell is sufficient to prevent the aberrant growth of the CD23^{+} CD21^{mid} IgD^{lo} population.

Figure 8: Results from flow analysis of hCD37 transgenic mouse



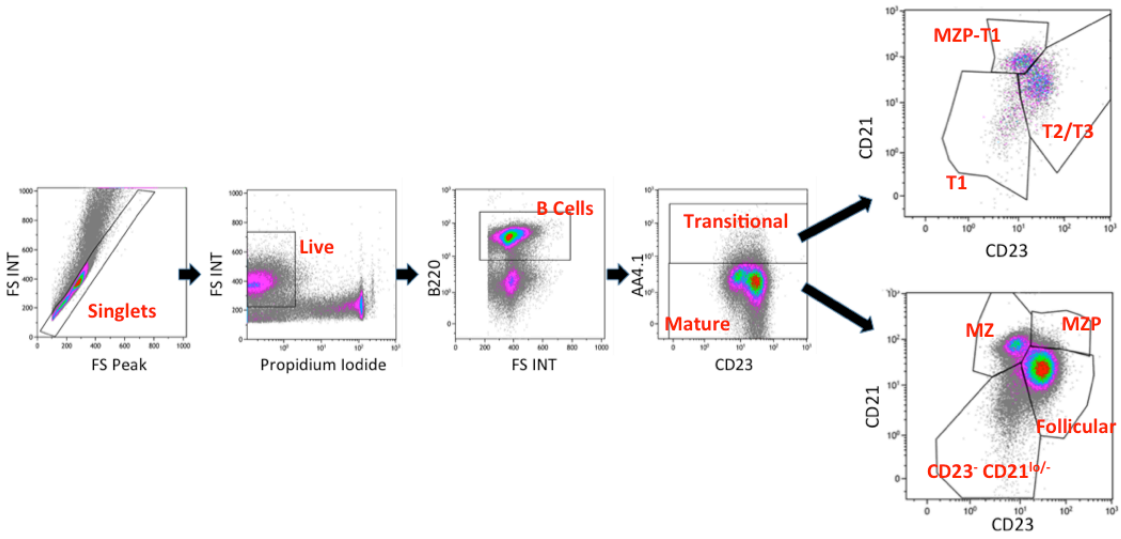
Bar graphs represent percentage of population out of total number of B cells ($B220^{+}$) collected in flow cytometric analysis of a human CD37 transgenic mouse, a hCD37 Tg Ets-$1^{-/-}$ mouse and an Ets-$1^{-/-}$ mouse.

Figure 9: Marginal Zone Progenitor to marginal zone transition requires a hematopoietic extrinsic role of Ets-1



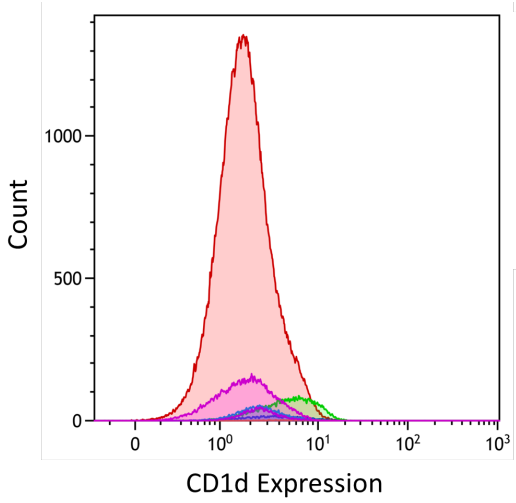
Ets-1 may play an extrinsic role at the MZP to MZ B cell transition and a hematopoietic intrinsic role after the T1 stage.

Figure S1: Gating strategy for analyzing various splenic B cell subpopulations



Splenocytes were incubated with different antibodies that were used to identify the various splenic B cell subpopulations. Live cells were gated based on forward scatter area (integral) and peak. Live B cells were identified by staining in Propidium Iodide (PI) and B220. AA4.1 was then used to differentiate between mature and immature B cells. The various splenic B cell subpopulations were then identified using CD21 and CD23.

Figure S2: Increased CD1d expression found in B cells along the MZ developmental pathway



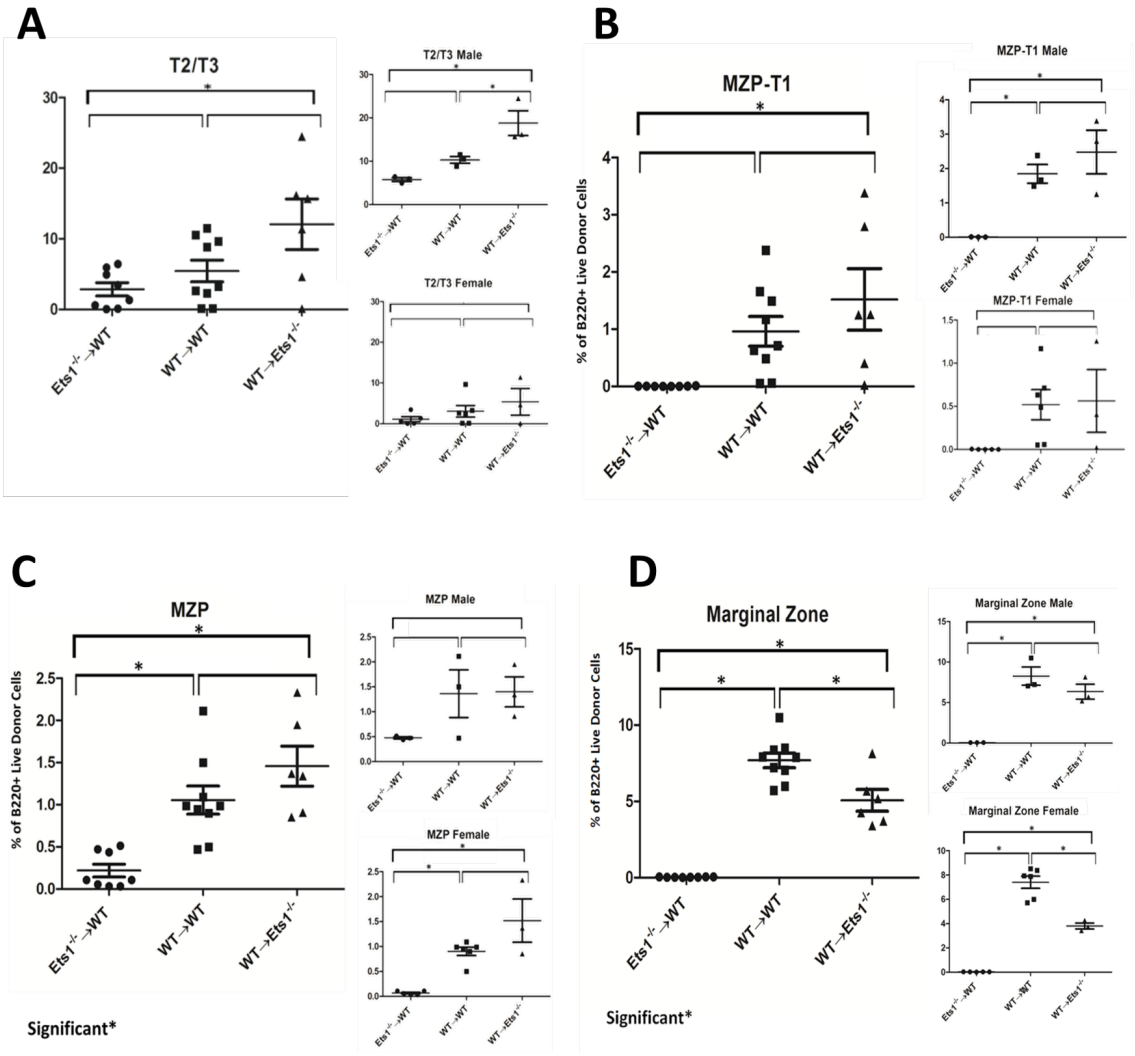
A

	Marker	X-Median
	Marginal Zone	5.16
	Marginal Zone Progenitor	3.52
	MZP-T1	5.39
	T1	2.51
	T2/T3	2.50
	Follicular	1.71
	$CD23^{-}CD21^{lo/-}$	1.70

B

A.) Histogram of CD1d expression across mature and immature splenic B cell subpopulations. Histogram overlay shows various subpopulations, as per the gating strategy shown in Figure 1. B.) Table shows the populations associated with each histogram on overlay (Fig 2A) and the corresponding X-Median measuring CD1d expression.

Figure S3: Adoptive transfer results from T2/T3. MZP-T1, MZP and Marginal Zone cell subpopulations.



When analyzing after three months, results suggested heavy intrinsic role of Ets-1 and some degree of extrinsic role as well. A.) Results suggest that the decrease in T2/T3 cells may be driven by a hematopoietic intrinsic effect through an observed increase in the WT→Ets-$1^{-/-}$ mouse compared to the irradiation control. Since, when there is Ets-1 present in the host, less development of T2/T3 cells is seen and when there is Ets-1 present in the donated bone marrow cell more T2/T3 cell is seen, it is possible that Ets-1 plays a negative regulatory role in the non-

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